

REMARKS

Applicants respectfully request entry of the above amendments and reconsideration of the following arguments pursuant to 37 C.F.R. § 1.111.

1. Amendments to Specification and Substitute Sequence Listing

Applicants amend the Specification to label the recited *E. coli* OmpT protease with a corresponding sequence identifier. Applicants also provide herewith a Substitute Sequence Listing, and a Declaration under 37 C.F.R. §§ 1.821-1.825. Applicants respectfully request entry of the presently submitted Sequence Listing.

Applicants submit that the amendments to the Specification have not introduced any prohibited new matter, because they are at least supported by the Specification as filed.¹

2. Status of the Claims

The status of the claims following entry of the amendments is as follows:

Claims canceled: 1-48, 50, 57-58, and 63-66
Claims pending: 49, 51-56, 59-62, and 67-68
Claims rejected: 49, 51-56, 59-62, and 67-68
Claims amended: 49, 51, and 55-56

3. Support for Amendments

Applicants amend claims 49, 51, and 55-56 to more precisely recite the claimed subject matter. Support for the claims amendments can be found at least from the claims previously presented and throughout the Specification. For example, the recitation of “*E. coli* OmpT protease variant consisting of an amino acid substitution at the 97th position of the amino acid sequence of SEQ ID NO: 41” can be found at least from (1) paragraph bridging pages 2-3; and

¹ Applicants submit that SEQ ID NO: 41 is the amino acid sequence of the mature form of *E. coli* OmpT protease, the sequence of which was identified in Sugimura et al., 170 J. BACTERIOL. 5625 (1988). See also paragraph bridging pages 2-3, and the paragraph bridging pages 7-8 of the Specification. SEQ ID NO: 40 is the amino acid sequence of the precursor of *E. coli* OmpT protease (having the signal sequence intact), while SEQ ID

(2) the paragraph bridging pages 7-8 of the Specification. Applicants do not believe that the amendments add prohibited subject matter that is unsupported by the as-filed Specification.

The claims have been amended without prejudice to, or disclaimer of, the canceled subject matter. Applicants reserve the right to file a continuation or divisional application on any subject matter canceled by way of amendments.

4. Interview Summary

Applicants appreciate the in-person interview conducted on May 17, 2010, between Examiners Sheridan L. Swope and Robert B. Mondesi and Applicants' representatives. During the interview, Applicant's representatives discussed with the Examiners the merits of each objection / rejection in view of the Amendment / Response filed March 15, 2011. The Examiners admitted that (1) Applicants' amendments would overcome at least some rejections, e.g., the rejection under 35 U.S.C. §§ 101 and 102; and (2) the cancellation of claims 36-48, 50, 57-58, and 63-66 would render some aspects of the other rejections mooted, as acknowledged with the withdrawal of the rejections.

5. Acknowledgement of Information Disclosure Statements

Applicants appreciate the Office's acknowledgement of the Information Disclosure Statement (IDS) filed November 15, 2007.

6. The Application is "Special"

Applicants appreciate (1) the Office's acknowledgement of the "special" status of the instant application, and (2) the involvement of the Supervisory Patent Examiner in the instant application.

NO: 39 is the nucleotide sequence encoding the *E. coli* OmpT protease precursor. See Sugimura et al., 170 J. BACTERIOL. 5625 (1988); see also GenBank M23630.1 and AAA24430.1.

7. **Priority**

Applicants attach hereto a verified English translation of Japanese priority application, JP 2003-342183. By perfecting priority with this translation, Applicants claim benefit of the filing date of September 30, 2003. *See* 37 C.F.R. § 1.55(a) and M.P.E.P. § 706.02(b).

8. **Withdrawn Objections and Rejections**

Rejections and objections not reiterated stand withdrawn. *See* 37 C.F.R. § 1.113(b); M.P.E.P. §§ 706.07 and 707.07(e).

9. **Rejection under 35 U.S.C. § 112, Second Paragraph**

The Office rejects claims 49, 51-56, 59-62, and 67-68 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Office Action, page 3.

9.1. **Claim 51**

The Office newly alleges that the recitation “wherein the amino acid sequence ... *consists of* a single basic amino acid or two or three consecutive basic amino acids” render claim 51 indefinite, because the recited amino acid sequence allegedly contains eight (8) amino acids. *Id.* The Office suggests that the recitation should be amended to “... *comprises only* a single basic amino acid or *only* two or three consecutive basic amino acids.” *Id.*, at 3-4. Applicants appreciate the Office’s suggestion and amend claim 51 accordingly. Applicants respectfully request withdrawal of the rejection and allowance of claim 51.

9.2. **Claims 49, 51-55, 59-62, and 67-68**

The Office maintains that the recitation of “a P10 position” and “a P3 position” in both claims 51 and 55 should be corrected to “the P10 position” and “the P3 position.” *Id.*, at 4. Applicants appreciate the Office’s suggestion and amend claims 51 and 55 accordingly.

Applicants also amend claim 49 to recite “wherein the polypeptide comprises a cleavage site that is a peptide bond between *the* P1 position and *the* P1’ position.” (emphasis added). Applicants submit that there is sufficient antecedent basis for reciting “the P1 position” and “the

P1' position" in claim 49, because the recited "cleavage site" inherently would have referred to the "P1 position" and "P1' position." See, e.g., *Bose Corp. v. JBL, Inc.*, 274 F.3d 1354, 1359, 61 U.S.P.Q.2d 1216, 1218-19 (Fed. Cir. 2001) (holding that inherent components of elements recited have antecedent basis in the recitation of the components themselves); see also M.P.E.P. § 2173.05(e).

Applicants respectfully request withdrawal of the rejection and allowance of the claims.

10. Rejection under 35 U.S.C. § 112, First Paragraph (Written Description)

The Office maintains the rejection of claims 49, 51-56, 59-62, 67 and 68 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. Office Action, page 4.

10.1. The Recited *E. coli* OmpT Protease Variants

The Office alleges that "the instant claims encompass any variant of said *E. coli* proteins having any structure comprising a substitution of a residue corresponding to Asp97 of the *E. coli* OmpT protease of Sugimura et al., 1988a (AAA24430.1)." *Id.* at 5. The Specification allegedly "fails to disclose using any variant of any protein other than the *E. coli* OmpT protease of Sugimura et al., 1988a (AAA24430.1)." *Id.*

Without acquiescing as to the merits of the Office's rejection, Applicants amend the claims to recite "an *E. coli* OmpT protease variant consisting of an amino acid substitution at the 97th position of the amino acid sequence of SEQ ID NO: 41." The claimed processes are directed to a variant of *E. coli* OmpT protease (parent) having the amino acid sequence of SEQ ID NO: 41, wherein the 97th amino acid of the mature *E. coli* OmpT protease (*i.e.*, Asp⁹⁷) is replaced with the recited amino acid (D97L, D97H, and D97M). Given the amendments, the Office rejection is mooted. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

10.2. The Recited Cleavage Sites

Grounds For Rejection

The Office alleges that “the instant claims encompass using any said variant to cleave an essentially unlimited number of substrates.” Office Action, page 5. The Specification allegedly fails to adequately describe the encompassed scope of cleavage methods. *Id.* Relying on the Specification, the Office also alleges that “cleavage of Arg↓Arg, and other P1↓P1’ motifs, by OmpT variants is dependent on residues outside of the P1 and P1’ positions and remained unpredictable.” *Id.*, at 6. The Office concludes that “the full scope of variant proteases and substrates thereof is not described such that the skilled artisan would recognize possession.” *Id.*, at 7.

Arguments

A. The Office Misinterprets The Claimed Processes

First, the claimed processes do not encompass “*any* variant of said *E. coli* proteins having any structure comprising a substitution of a residue corresponding to Asp97 of the *E. coli* OmpT protease.” Instead, the claimed processes are directed to a variant of *E. coli* OmpT protease (parent), wherein the 97th amino acid of the mature *E. coli* OmpT protease (*i.e.*, Asp⁹⁷) is replaced with the recited amino acid (D97L, D97H, and D97M). *See* section 10.1 *supra*.

Additionally, the claims recite *inter alia* cleaving a polypeptide with an *E. coli* OmpT protease variant having the recited amino acid sequence, wherein the polypeptide comprises a cleavage site (P1↓P1’), and wherein the variants and the cleavage sites are arranged according to the table below:

	The 97 th Amino Acid of the Variant	Cleavage Site	
		P1	P1’
I	Leucine (D97L)	Arginine or Lysine	Serine or Alanine
II	Methionine (D97M)	Arginine or Lysine	Phenylalanine, Alanine, Serine, Cysteine, or Tyrosine
III	Histidine (D97H)	Arginine or Lysine	Alanine, Valine, Isoleucine, Methionine, Serine, Threonine, Cysteine, or Asparagine

The claims are directed to “cleaving” polypeptides having the recited motifs. Giving the claims their broadest reasonable interpretation consistent with the specification and with the interpretation the skilled artisan would reach, the claim reads on cleaving with *any degree of efficiency*. See, e.g., *In re Morris*, 127 F.3d 1048, 1054, 44 U.S.P.Q.2d 1023, 1027 (Fed. Cir. 1997). Amino acid residues outside the P1↓P1’ may at best affect the cleavage *efficiency*. Applicants submit that the present claims do not recite cleavage efficiency. Accordingly, the alleged unpredictability is irrelevant in determining whether the present claims comply with the written description requirement.

B. The Office’s Position as to Working Examples are Unsupported

Furthermore, the Office’s assertion as to inadequacy of working examples (“functional permutations”) is unsupported. Working examples covering the full scope of the claims are not required for an adequate written description. See e.g., *Falkner v. Inglis*, 448 F.3d 1357, 1366, 79 U.S.P.Q.2d 1001, 1007 (Fed. Cir. 2006);² see also *Ariad Pharmaceuticals Inc. v. Eli Lilly & Co.*, 598 F.3d 1336, 1352, 94 U.S.P.Q. 1161, 1172 (Fed. Cir. 2010) (*en banc*).³ “[I]t is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention.” See *Capon v. Eshhar*, 418 F.3d 1349, 1359, 76 U.S.P.Q.2d 1078, 1085 (Fed. Cir. 2005) (citing *In re Angstadt*, 537 F.2d 498, 504, 190 U.S.P.Q. 214, 218 (C.C.P.A. 1976)); see also *Ariad*, 598 F.3d at 1351, 94 U.S.P.Q.2d at 1172 (citing *Capon* with approval).

The Specification provides multiple working examples, wherein the claimed OmpT variants (D97L, D97H, and D97M) cleave various motifs within the scope of the claims. See, e.g., Specification, Examples 13-18 and Table 1. While it is possible that some motifs encompassed by the claims may not be cleaved, there is no requirement under relevant U.S. judicial precedent to show reduction to practice of every permutation within the claims. See

² “A claim will not be invalidated on section 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before.”

³ “We have made clear that the written description requirement does not demand either examples or an actual reduction to practice; a constructive reduction to practice that in a definite way identifies the claimed invention can satisfy the written description requirement.”

Angstadt, 537 F.2d at 504, 190 U.S.P.Q. at 218. Instead, the Specification need only sufficiently characterize the generic invention. Applicants hereby submit that the Specification adequately describes the claimed cleavage sites.

Given at least the above arguments, the Office's rejection is unsupported. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

11. Rejections under 35 U.S.C. § 102(a)

The Office rejects claims 49 and 51-55, 59-61, and 67-68 under 35 U.S.C. § 102(a) as allegedly anticipated by **Okuno et al.**, 70 APPL. ENVIRON. MICROBIOL. 76 (2004) ("Okuno 2004"). Office Action, page 7. Okuno 2004 allegedly discloses the recited OmpT protease variants and the recited cleavage sites. *Id.*, at 7-8.

With the submission of a verified English translation of JP 2003-342183, Applicants have perfected priority to the instant Japanese application and the filing date of September 30, 2003. See Section 6 Priority, *supra*. The perfected filing date antedates the publication date of Okuno 2004—January 2004. Thus, Okuno 2004 is no longer available as a prior art reference. The Office's rejection is mooted. Applicants respectfully request withdrawal of the rejection and allowance of the claims.

12. Rejections under 35 U.S.C. § 103(a)

12.1. Claim 56

The Office newly rejects claim 56 under 35 U.S.C. § 103(a) as allegedly obvious over **Okuno 2004** in view of **Lejal et al.**, 81 J. GEN VIROL. 983 (2000) ("Lejal"). Office Action, page 8.

Grounds For Rejection

The alleged teachings of Okuno 2004 are discussed above. The Office admits that Okuno 2004 "does not teach a method of cleaving a polypeptide by coexpressing the polypeptide with the OmpT protease variant." *Id.* Lejal allegedly teaches "cleaving a polypeptide by coexpressing the polypeptide with a protease." *Id.* It allegedly "would have been obvious to a person of ordinary skill in the art to combine the teachings of Okuno et al and Lejal et al to make a method

for cleaving fusion proteins using variant of OmpT protease ... wherein the fusion protein and variant are co-expressed in host cells.” *Id.*

Arguments

Applicants traverse. “[O]bviousness requires a suggestion of *all* limitations in a claim.” *CFMT, Inc. v. Yieldup Int’l Corp.*, 349 F.3d 1333, 1342, 68 U.S.P.Q.2d 1940, 1947 (Fed. Cir. 2003) (emphasis added). Once the scope and content of the prior art are determined, the relevant inquiry is whether the prior art suggests the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful. *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991).

Claim 56 is nonobvious for at least the following reasons. First, Okuno 2004 is no longer available as a primary reference, because Applicants have perfected priority to September 20, 2003. *See* Section 7 Priority, *supra*. Applicants submit that Lejal alone does not render claim 56 obvious. Claim 56 recites, *inter alia*, cleaving a polypeptide with an *E. coli* OmpT protease variant, wherein the variants and the cleavage sites are arranged as recited. Lejal is relied upon for its purported teaching of co-expressing a protease and its targeting polypeptide in a host cell. Lejal does not teach using an *E. coli* OmpT protease variants to cleavage polypeptides having the recited cleavage sites. Thus, Lejal fails to teach or suggest all claim elements. Without all claim elements taught, there can be no expectation to practice the claimed processes predictably.

Given at least these arguments, claim 56 is nonobvious over cited references. Applicants respectfully request withdrawal of the rejection and allowance of claim 56.

12.2. Claims 49 and 51-53

Claims 49 and 51-53 over **Okuno et al.**, 66 BIOSCI. BIOTECHNOL. BIOCHEM. 127 (2002) (“Okuno 2002a”), **Dekker et al.**, 40 BIOCHEMISTRY 1694 (2001) (“Dekker”), **Kramer et al.**, 505 FEBS LETTERS 426 (2001) (“Kramer”) in view of **Metzler**, BIOCHEMISTRY, 2nd ed., Harcourt / Academic Press (2001) (“Metzler”). Office Action, page 8.

Grounds For Rejection

The Office admits that the combination of the references “does not teach cleavage of one or more said substrate with an OmpT protease variant having a substitution at Asp⁹⁷ with an

amino acid other than Ala.” *Id.*, at 9. Kramer allegedly teaches that “the residue at position 97 of OmpT protease determines substrate specificity in regards to position P1’”. *Id.* A skilled artisan allegedly would have been motivated to make OmpT protease variants “at Asp⁹⁷ with each of the naturally occurring amino acids, including **Leu** and **Met**.” The Office further alleges that “the skilled artisan would have believed that, more likely than not the OmpT protease variants having a substitution at Asp⁹⁷ with **Leu** or **Met** would cleave a substrate comprising a P1’ that is S, A, F, C, Y, or V.” *Id.* (emphasis original).

Arguments

Applicants traverse. The framework of determining obviousness under 35 U.S.C. § 103 must rely on the framework for an *objective analysis*. See, e.g., *Graham v. John Deere Co.*, 383 U.S. 1, at 17-18, 148 U.S.P.Q. 459, 467 (1966) (*accord KSR Int’l Co. v. Teleflex Inc.*, 550 U.S. 398, 82 U.S.P.Q.2d 1385 (2007)). The objective analysis requires that the Office take the perspective *from a skilled artisan at the time*. *Id.*; see also M.P.E.P. § 2141. To establish *prima facie* obviousness using a combination of multiple references, the Office must show that the combination or modification must have expected and predictable results. See M.P.E.P. § 2143.

A. The Office Fails To Objectively Apply Kramer’s Teachings

First, the Office exaggerates Kramer’s teachings as to the role of Asp⁹⁷ in the P1’ specificity of OmpT protease. Kramer does *not* teach that Asp⁹⁷ of the OmpT protease determines its P1’ specificity. Applicants direct the Office to lines 2 to 8, left col., at page 429 of Kramer:

Assuming that the substrate has an extended conformation and that the P1 site chain points toward Glu²⁷ and Asp²⁰⁸, the P1’ chain *would be* located close to Asp⁹⁷. D97A OmpT displayed only 6% residual activity, therefore we *propose* that Asp⁹⁷ is responsible for the observed P1’ specificity.

Given the above description, a skilled artisan would have understood that Kramer merely *speculates* as to the role of Asp⁹⁷ in the P1’ specificity. Given such a speculation, there can be no reasonable expectation that the replacement of Asp⁹⁷ with other amino acid(s) would have changed substrate specificity, let alone the presently claimed amino acid substitutions (D97L, D97H, and D97M) of Asp⁹⁷. The Office’s rejection does not rest on the framework of an objective analysis—Kramer does not teach what the Office asserts, and is thus unsupported.

B. There Is No Motivation To Make Or Use The Claimed Variants

Furthermore, a skilled artisan would not have been motivated to make and use the claimed *E. coli* OmpT protease variants given the knowledge in the field at the time. The Office is respectfully directed to **Olsen** et al., 18 NATURE BIOTECHNOL. 1071 (2000) (“Olsen”) (enclosed as **EXHIBIT I**). One of the research objectives of Olsen is to produce OmpT variants with novel or expanded substrate specificities (*e.g.*, Arg↓Val, toward which the “wild-type OmpT protease possesses a modest ability to cleave”). *See* Olsen, paragraph bridging columns, at page 1072. To achieve these objectives, Olsen generated a library of OmpT variants by random mutagenesis, and assayed for variants with increased activity against various substrates. *See, id.*, left columns, at page 1074. Olsen’s screening identified three OmpT variants having enhanced cleavage rates for Arg↓Val. *See, id.*, Table 1 at page 1072. Each variant contains multiple substitutions. *See, id.*, Table 2 at page 1073. However, none of the substitutions is at the 97th amino acid residue (Asp⁹⁷) of OmpT protease. *Id.*

Given the above teachings, a skilled artisan at the time would have focused on the residues identified in Olsen to produce additional OmpT variants having expanded substrate specificity. Alternatively, given the success of Olsen’s methodology (*i.e.*, random mutagenesis coupled with high-throughout screening), a skilled artisan may have performed independent random mutagenesis experiments to produce additional OmpT variants having expanded substrate specificity. There is no evidence that such screening may have identified Asp⁹⁷ of *E. coli* OmpT protease. Also, Olsen provides no guidance to focus on Asp⁹⁷. Given Olsen, a skilled artisan at the time would *not* have been motivated to make and test any *E. coli* OmpT protease variant having a substitution at the 97th position. There is no evidence on the record or adduced by the Office why a skilled artisan would have ignored the teachings of Olsen to adopt an approach (without any direction to do so) without a reasonable expectation of success. *See supra.*

C. There Is No Motivation To Achieve The Claimed Combinations of Variants and P1/P1’ Cleavage sites

The Office also fails to explain why a skilled artisan at the time would have been motivated to achieve the claimed combinations between the claimed *E. coli* OmpT protease

variants and the claimed cleavage sites. The claims recite *inter alia* cleaving a polypeptide with an *E. coli* OmpT protease variant having the recited amino acid substitution (D97L, D97H, or D97M), wherein the combinations between the claimed 97th amino acid substitution of the *E. coli* OmpT protease variant and the claimed cleavage site (P1↓P1') are further elucidated below:

	The 97 th Amino Acid of the Variant	Cleavage Site	
		P1	P1'
1	Leucine (D97L)	Arginine	Serine
2		Arginine	Alanine
3		Lysine	Serine
4		Lysine	Alanine
5	Methionine (D97M)	Arginine	Phenylalanine
6		Arginine	Alanine
7		Arginine	Serine
8		Arginine	Cysteine
9		Arginine	Tyrosine
10		Lysine	Phenylalanine
11		Lysine	Alanine
12		Lysine	Serine,
13		Lysine	Cysteine
14		Lysine	Tyrosine
15	Histidine (D97H)	Arginine	Alanine
16		Arginine	Valine
17		Arginine	Isoleucine
18		Arginine	Methionine
19		Arginine	Serine
20		Arginine	Threonine
21		Arginine	Cysteine
22		Arginine	Asparagine
23		Lysine	Alanine
24		Lysine	Valine
25		Lysine	Isoleucine
26		Lysine	Methionine
27		Lysine	Serine
28		Lysine	Threonine
29		Lysine	Cysteine
30		Lysine	Asparagine

Accordingly, the claimed methods only encompass altogether the above-enumerated thirty (30) combinations. The claimed combination set is only a small fraction (less than 1/250 or 0.4%) of the total possible combinations.⁴ Even if it were assumed *arguendo* that a skilled artisan were motivated to test the cleavage by a *E. coli* OmpT variant having an amino acid substitution at the 97th position (which it is not), there is no evidence on the record or adduced by the Office that a skilled artisan would have reached the presently claimed set of the combinations among all possible combinations.

Given at least the above arguments, the Office's rejection is unsupported. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

12.3. Claims 49 and 51-53

The Office newly rejects claims 49 and 51-53 as allegedly obvious over the combination of **Okuno 2002a, Dekker, and Kramer** in view of **Wolf et al**, 270 J. BIOL. CHEM. 16097 (16097) ("Wolf"). Office Action, page 14.⁵

Grounds For Rejection

By relying on Kramer, the Office alleges that a skilled artisan allegedly would have been motivated to make OmpT protease variants "at Asp⁹⁷ with each of the naturally occurring amino acids, including **His**." The Office also alleges that "the skilled artisan would have believed that, more likely than not the OmpT protease variants having a substitution at Asp⁹⁷ with **His** would cleave a substrate comprising a P1' that is S, T, or R." *Id.*, at 15 (emphasis original). Wolf

⁴ There could be altogether more than seven thousand (> 7000) combinations between the 97th position substitution and the cleavage site (P1↓P1'). This number is calculated as 19 (number of amino acids other than Asp as the 97th amino acid of an OmpT protease variant) × 20 (number of amino acids for the P1 position of the cleavage site) × 20 (number of amino acids for the P1' position of the cleavage site) = 7600.

⁵ The Office alleges:

Regarding the above rejection of Claims 49 and 51-53 over the combination of Okuno et al, 2002a, Dekker et al, 2001, and Kramer et al, 2001 in view of *Wolf et al, 1995*, Applicants provided the same arguments, as set forth above regarding the rejection of Claims 49 and 51-53 are rejected under 35 U.S.C. 103(a) as being unpatentable over the combination of Okuno et al, 2002a, Dekker et al, 2001, and Kramer et al, 2001 in view of Metzler, 2001. Said arguments are not persuasive for the reasons set forth above.

Office Action, page 15 (emphasis added). Applicants submit that the Office newly applies Wolf in the Office Action mailed July 7, 2011 (cited in the PTO-892 form). Applicants' prior arguments have never addressed Wolf. For the record, Applicants respectfully request the Office clarification in this regard with its next communication.

allegedly teaches that “binding of histidine to HisJ is mediated by binding with S, T, and R residues.” *Id.*

Arguments

Applicants traverse. Okuno 2002a, Dekker, and Wolf, alone or in combination, do not teach the claimed *E. coli* OmpT variants. The Office entirely relies on Kramer’s D97A variant to assert a skilled artisan’s motivation to make additional amino acid substitutions at the 97th position of OmpT protease to create additional variants. For similar reasons as discussed in section 12.2 *supra*, the Office’s rejection is unsupported for failing to rest on a framework of an objective analysis—Kramer does not teach what the Office asserts. There also can be no reasonable expectation that the replacement of Asp⁹⁷ with other amino acid(s) would have changed substrate specificity, let alone the presently claimed amino acid substitutions (D97L, D97H, and D97M) of Asp⁹⁷. Similarly, a skilled artisan at the time would *not* have been motivated to (1) make and test any *E. coli* OmpT protease variants having an amino acid substitution at the 97th position given Olsen’s teachings; and (2) reach the claimed combinations of the amino acid substitution and the cleavage site as argued in Section 12.2 *supra*.

Given at least the above arguments, the Office’s rejection is unsupported. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

12.4. Claims 54-56, 59-61, and 67-68

The Office maintains the rejection of claims 54-56, 59-61, and 67-68 over **Stumpe et al.**, 180 J. BACTERIOL. 4002 (1998) (“Stumpe”), **Suzuki et al.**, 72 J. BIOCHEM. 1419 (1972) (“Suzuki”), **Kramer, Yamamoto et al.**, U.S. Patent No. 5,506,120 (“Yamamoto”), and **Metzler**. Office Action, pages 15-16.

Grounds For Rejection

By relying on Kramer, the Office alleges that a skilled artisan allegedly would have been motivated to make OmpT protease variants “at Asp⁹⁷ with neutral/hydrophobic amino acids, including Ala, Val, Ile, Phe, Tyr, and Trp, **Met** and **Leu**.” The Office also alleges that “the skilled artisan would have believed that, more likely than not the OmpT protease variants having a

substitution at Asp⁹⁷ with Met would cleave a substrate comprising a P1' of F." *Id.*, at 16 (emphasis original).

Arguments

Applicants traverse. Stumpe, Suzuki, Yamamoto, and Metzler, alone or in combination, do not teach the claimed *E. coli* OmpT protease variants. The Office entirely relies on Kramer's D97A variant to assert a skilled artisan's motivation to make additional amino acid substitutions at the 97th position of OmpT protease to create additional variants (e.g., D97M). For similar reasons as discussed in section 12.2 *supra*, the Office's rejection is unsupported for failing to rest on a framework of an objective analysis—Kramer does not teach what the Office asserts. There also can be no reasonable expectation that the replacement of Asp⁹⁷ with other amino acid(s) would have changed substrate specificity, let alone the presently claimed amino acid substitution (D97M) of Asp⁹⁷. Similarly, a skilled artisan at the time would *not* have been motivated to (1) make and test any *E. coli* OmpT protease variants having an amino acid substitution at the 97th position given Olsen's teachings; and (2) reach the claimed combinations of the amino acid substitution and the cleavage site as argued in Section 12.2 *supra*.

Given at least the above arguments, the Office's rejection is unsupported. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

CONCLUSION

In view of the above arguments and amendments to the claims, Applicants submit that the claims are in condition for allowance and respectfully request reconsideration and timely allowance of the claims.

Should the Office have any questions or comments regarding Applicants' amendments or response, please contact Applicants' undersigned representative at (202) 230-5119. Furthermore, please direct all correspondence to the below-listed address.

In the event that the Office believes that there are fees outstanding in the above-referenced matter and for purposes of maintaining pendency of the application, the Office is authorized to charge the outstanding fees to Deposit Account No. 50-0573. The Office is likewise authorized to credit any overpayment to the same Deposit Account Number.

Dated: October 7, 2011

Respectfully submitted,

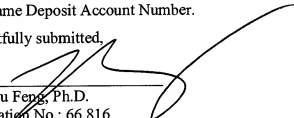
By 
Zhengyu Feng, Ph.D.
Registration No.: 66,816
DRINKER BIDDLE & REATH LLP
1500 K Street, N.W., Suite 1100
Washington, DC 20005-1209
202.230.5119
202.842.8465 (Fax)
Attorneys/Agents For Applicant

EXHIBIT I

Olsen et al., 18 NATURE BIOTECHNOL. 1071 (2000)

Function-based isolation of novel enzymes from a large library

Mark J. Olsen¹, Daren Stephens^{2,3}, Devin Griffiths³, Patrick Daugherty^{2,4}, George Georgiou^{2,3*}, and Brent L. Iverson^{1,3*}

¹Department of Chemistry and Biochemistry, ²Department of Chemical Engineering, and the ³Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712. ⁴Present address: Fred Hutchinson Cancer Research Center, Division of Molecular Medicine, P.O. Box 19024, Seattle, WA 98109-102. *Corresponding authors. B.L.I. (biverson@utvms.cc.utexas.edu) and G.G. (gg@che.utexas.edu).

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Here we describe a high-throughput, quantitative method for the isolation of enzymes with novel substrate specificities from large libraries of protein variants. Protein variants are displayed on the surface of microorganisms and incubated with a synthetic substrate consisting of (1) a fluorescent dye (2) a positively charged moiety (3) the target scissile bond, and (4) a fluorescence resonance energy transfer (FRET) quenching partner. Enzymatic cleavage of the scissile bond results in release of the FRET quenching partner while the fluorescent product is retained on the cell surface, allowing isolation of catalytically active clones by fluorescence-activated cell sorting (FACS). Using a synthetic substrate with these characteristics, we enriched *Escherichia coli* expressing the serine protease OmpT from cells expressing an inactive OmpT variant by over 5,000-fold in a single round. Screening a library of 6×10^5 random OmpT variants by FACS using a FRET peptide substrate with a nonpreferred Arg-Val cleavage sequence resulted in the isolation of variant proteases with catalytic activities enhanced by as much as 60-fold. This approach represents a potentially widely applicable method for high-throughput screening of large libraries on the basis of catalytic turnover.

Keywords: Flow cytometry, directed enzyme evolution, fluorescence

Directed evolution of a desired catalytic activity from libraries of random mutants represents a powerful route to enhancing the stability and substrate specificity of enzymes^{1,2}. Mutant library construction using targeted random mutagenesis and/or DNA shuffling followed by colony-based plate assays has led to the isolation of remarkable enzyme variants from relatively small libraries, typically containing 10^3 – 10^4 members^{3–5}. Rapid screening of very large libraries, however, has been possible for only a few enzymatic reactions in which the desired function can be linked to a selectable phenotype^{6–11}. Phage display offers a possible alternative to colony-based plate screening approaches because it is amenable to screening large libraries; however, enrichment is generally based on ligand binding rather than catalytic turnover^{12–15}.

Herein we present a new strategy for the catalytic turnover-based isolation of desired enzyme variants from large libraries. As shown in Figure 1, enzyme variants are displayed on the cell surface of microorganisms¹⁶, giving them free access to substrates. We synthesized a fluorescence resonance energy transfer (FRET) substrate consisting of (1) a fluorophore (Fl), (2) a moiety with a +3 charge, (3) the scissile bond, and (4) a quenching fluorophore that acts as an intramolecular FRET partner (Q). Because the surface of *Escherichia coli* is negatively charged (ζ potential –25 to –30 mV)¹⁷, the positively charged FRET substrate associates with the cell surface. The Fl and Q moieties are then separated by enzymatic cleavage of the scissile bond, resulting in disruption of intramolecular FRET quenching.

Using such cell surface-retained substrates and reaction products allowed us to link an enzyme-encoding gene to catalytic turnover. We then analyzed cells for fluorescence in real time and with high throughput using multiparameter flow cytometry^{18–21}. Clones exhibiting high catalytic activities were enriched by fluorescence-activated cell sorting (FACS), which resulted in the isolation of a small number of catalytically active clones that are easily analyzed in detail using robotic 96-well plate assays.

The *E. coli* OmpT (EC 3.4.21.87), a surface-displayed protease, is the prototypical enzyme of the omptin family that includes several serine proteases implicated in microbial pathogenicity^{22,23}. Examination of the cleavage specificity of OmpT using substrate phage revealed a strong preference for basic residues (Arg, Lys) in both the P1 and P1' subsites, a specificity similar to the prohormone convertases (J.J. McCarter, D.L. Stephens, G. Georgiou and J.F. Kirsch, unpublished data). Consistent with earlier reports^{24,25}, OmpT was found to be an efficient enzyme, catalyzing the hydrolysis of optimal peptide substrates such as AcWGGERIKGWGT-CONH₂ with an efficiency of $k_{cat}/K_M = 3.3 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$ (ref. 22). Because of its well-characterized cleavage specificity, ease of purification, and natural localization on the cell surface, we used OmpT for the present studies.

Results and discussion

Substrate design. The FRET substrate 1 (Fig. 2) contains the BODIPY (Fl) and tetramethylrhodamine (Q) fluorophores on either side of an Arg-Arg sequence optimal for OmpT cleavage. Substrate 1 has an overall +3 charge that promotes electrostatic binding to the *E. coli* surface in low-ionic-strength solutions. The low-ionic-strength buffers used contain sucrose to avoid plasmolysis. Hydrolysis of substrate 1 by OmpT at the Arg-Arg peptide bond gives rise to a C-terminal product containing the BODIPY (Fl) fluorophore on a short peptide with a +3 overall charge (two arginine guanidinium groups as well as the unmasked N-terminal amine). In contrast, the N-terminal product containing the tetramethylrhodamine (Q) fluorophore has no net charge, and presumably diffuses away from the cell.

Using substrate 1, FACS analysis can discriminate between three different cell populations: (1) cells with no OmpT on the surface, (2) cells with active OmpT catalysts on the surface, and (3) cells expressing an impaired OmpT variant. In particular, incubation of the *ompT*

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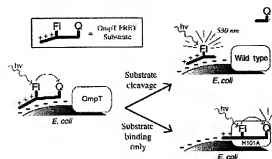


Figure 1. FRET substrate binding and fluorescence emission upon catalytic turnover or binding to protein displayed on the cell surface. FI, BODIPY; Q, tetramethylrhodamine.

strain UT5600 with substrate 1 results in baseline emission at 530 \pm 30 nm (FL1; from BODIPY) and 585 \pm 42 nm (FL2; from tetramethylrhodamine) wavelengths (population 1 in Fig. 3A). However, UT5600 cells transformed with the plasmid pML19, a pUC derivative expressing OmpT from its native promoter (D. Stephens & G. Georgiou, unpublished data), exhibit over 50-fold greater FL1 fluorescence, consistent with cleavage and cell surface product capture (population 3 in Fig. 3A). Cells transformed with a pML19 derivative encoding an OmpT (H101A) variant with approximately 4% of the specific activity of the wild-type enzyme (D. Stephens & G. Georgiou, unpublished data) exhibit a strikingly different fluorescence profile, characterized by moderate FL1 fluorescence but 20-fold greater FL2 fluorescence (population 2 in Fig. 3A). This increased FL2 emission could be due to the tetramethylrhodamine fluorophore being sequestered inside a protein-binding pocket, away from the solvent water molecules that would ordinarily limit emission resulting from collisional quenching.

Apparently substrate 1 partitions onto the cell surface, because a concentration of only 50 nM was sufficient to give the population discrimination shown in Figure 3A. The fluorescence profiles of the three cell populations remained virtually unchanged for at least 30 min of incubation with substrate 1, indicating that catalytic turnover and product capture rapidly reach steady state.

Catalytic enrichment. A 5,000-fold enrichment of clones expressing active catalysts was obtained in a single round using the FRET/FACS system. For these studies, we used cells in which the expression of OmpT was reduced by using the lower copy number vector pML319 (pBR322 origin of replication), which displays about four-fold lower FL1 fluorescence relative to expression from the higher copy number vector pML19 (pUC replication origin) when incubated with substrate 1 under identical conditions. In the enrichment experiment, UT5600/pML319 was mixed with a 5,000-fold excess of UT5600/pDS327, a pML319 derivative encoding a H212A mutation that results in a reduction in catalytic activity by about 10^3 (D. Stephens & G. Georgiou, unpublished data). The mixed cells were incubated with 1, and clones exhibiting high fluorescence were isolated by FACS, then grown in liquid media. Consistent with the expected frequency of active clones in the mixture, 32 highly fluorescent cells were detected in the positive window, out of approximately 150,000 bacteria analyzed. These 32 highly fluorescent cells were grown overnight; the culture (post-sort) was then labeled with substrate 1 and analyzed by flow cytometry. The fluorescence profile of the post-sort population was essentially indistinguishable from that of UT5600/pML319 cells expressing wild-type OmpT. Nine of nine clones chosen at random from the selected population were shown to contain pML319 as expected.

Evolution of substrate specificity. An enhancement of the rate of cleavage of substrates containing the Arg-

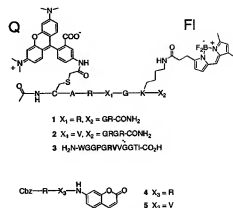


Figure 2. Substrates for the detection of catalytic activity.

Val sequence specificity was chosen as the target of initial directed-evolution experiments, because wild-type OmpT possesses a modest ability to cleave this sequence. In fact, the weak Arg-Val cleavage activity of OmpT is responsible for its role as a plasminogen activator, an function possibly related to bacterial pathogenicity²². Thus, peptide 3, a sequence derived from human plasminogen, is cleaved by OmpT at Arg-Val with a catalytic efficiency $k_{cat}/K_M = 24 s^{-1} M^{-1}$.

The FRET substrate 2, containing a putative Arg-Val cleavage site in the place of the Arg-Arg site in substrate 1, was synthesized and used to screen libraries of OmpT random variants. The additional Gly-Arg sequence in 2 compared to 1 was necessary to confer a +3 charge on the BODIPY-containing product. Because the optimal rate of random mutagenesis that must be used in order to obtain protein variants with altered function is not a priori obvious²³, the *ompT* gene was subjected to random mutagenesis by error-prone polymerase chain reaction (PCR) at different Mn^{2+} concentrations. The mutagenized DNA was amplified and then inserted within a high copy vector without ligation using the CloneAmp cloning strategy²⁴. A total of 6×10^6 transformants were obtained. The library was incubated with substrate 2 at 50 nM, and clones falling within an FL1-FL2 window expected for active clones were isolated by FACS. A total of 1.9×10^6 cells were evaluated in 24 min, and 352 individual clones were recovered, corresponding to an enrichment of 5,400-fold in one round (Fig. 3C).

The FACS-isolated clones were further ranked on the basis of hydrolysis of the simpler 7-amino-4-methylcoumarin (AMC) substrates 4 and 5 containing putative Arg-Arg and Arg-Val cleavage sites, respectively. These assays were carried out using a robotically implemented, coupled assay with aminopeptidase M. Cleavage of 4 and 5 by OmpT gives rise to H_2N -Arg-AMC and H_2N -Val-AMC,

Table 1. Catalytic efficiencies as well as cleavage products observed during cleavage of substrate 3 by OmpT and the three variants isolated in this study*

Enzyme	k_{cat}/K_M ($s^{-1}M^{-1}$) ^b	Cleavage site (%)		
		Arg-Val	Val-Val	Gly-Thr
OmpT	24	100	ND	ND
C5	1440	99	9	2
B4	310	32	35	12
G11	200	10	41	23

Substrate 3: $H-WGGPGRV-V-V-GG-TI-CONH_2$

Catalytic activities were monitored by HPLC. Identities of the individual cleavage products were determined by mass spectral analysis, and relative amounts were determined by HPLC. ND, None detected. For reference, the sequence of peptide substrate 3 is shown beneath the table, and the observed cleavage sites are indicated ().

^bThese apparent values were calculated by measuring rates of cumulative appearance of products during the cleavage reaction.

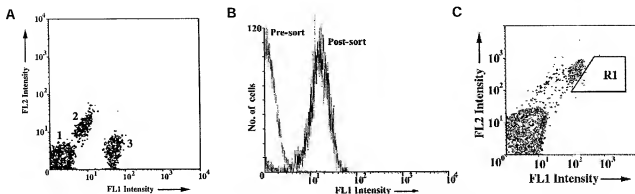


Figure 3. Flow cytometric discrimination of *E. coli* on the basis of OmpT activity. (A) UT5600 (population 1); pDS26 encoding OmpT H101A (population 2); pML19 encoding the wild-type OmpT (population 3). (B) Single-step enrichment of cells with wild-type OmpT (UT5600/pDS319) from a 5,000-fold excess of cells expressing the OmpT H212A variant (UT5600/pDS327). (C) Library data obtained when sorting OmpT library for increased RV cleavage activity using FRET substrate 2. The sort gate used to isolate 352 clones is shown as region R1 in the figure.

respectively, which are not fluorescent. These products, however, unlike substrates 4 and 5 in which the N terminus is blocked by a carbonylbenzoyloxy (Cbz) group, are substrates for aminopeptidase M. Aminopeptidase M removes the amino acid, releasing the highly fluorescent AMC moiety. Three clones out of the selected 352 exhibited a high ratio of cleavage activity of substrate 2 relative to wild type. For comparison, only one out of 1,200 randomly selected clones from the pre-sort library population gave fluorescent product that was slightly above background when analyzed for hydrolysis of substrate 2.

Kinetic analysis. Purified OmpT²⁸ from all three clones was found to hydrolyze substrate 2 with rates at least threefold higher than purified, wild-type OmpT. Surprisingly, the major hydrolysis product generated by all three variants with substrate 2 was AMC, whereas the expected product H₂N-Val-AMC was produced in lesser amounts. The fraction of 2 converted to AMC ranged from 78% (clone B4) to >96% (clone G11). In contrast, hydrolysis of 2 by the wild-type enzyme gave only the expected H₂N-Val-AMC cleavage product, with no detectable AMC. Subsequent experiments confirmed that the three variants did not possess detectable amino peptidase activity with H₂N-Val-AMC as substrate. Thus, cleavage of the Val-AMC bond of the intact substrate 2, as opposed to sequential Arg-Val then H₂N-Val-AMC cleavage, was responsible for the formation of the AMC cleavage product.

In order to assess the cleavage activities of the three variants in the context of a peptide substrate, cleavage activity with substrate 3 was analyzed (Table 1). Peptide 3 corresponds to the cleavage sequence of human plasminogen, a known substrate of OmpT, which is cleaved predominantly at the Arg-Val sequence. The variants C5, B4, and G11 hydrolyzed peptide 3 with apparent k_{cat}/K_M values of $1.440 \times 10^4 \text{ M}^{-1}$, $310 \times 10^4 \text{ M}^{-1}$, and $200 \times 10^4 \text{ M}^{-1}$, respectively, corresponding to catalytic efficiencies that are 60-fold, 13-fold, and 9-fold better than the wild-type OmpT enzyme.

Mass spectrometry analysis was used to determine cleavage products of peptide 3 (Table 1). Variant C5 cleaved peptide 3 at predominantly the expected Arg-Val cleavage site, as did wild-type OmpT. Thus, the most active variant, C5, displayed a significantly higher level of catalytic activity, yet overall similar specificity, with this peptide compared to the wild-type OmpT.

DNA sequencing of the three variants revealed that C5, B4, and G11 had 11, 7, and 9 single-base changes, respectively, corresponding to 8, 4, and 6 amino acid substitutions (Table 2). There is little in the way of homology among the amino acid changes. In fact, none of the sub-

stitutions with B4 coincide with changes in either C5 or G11, even though the catalytic activity of B4 appears to be a hybrid of C5 and G11 activities. In general terms, results for these OmpT variants having relatively high numbers of mutations are consistent with previous investigations of antibody plasticity in which a surprising number of gain-of-function variants have been found in highly mutated libraries.²⁹

The fact that variant C5 displays 60-fold enhanced Arg-Val catalytic efficiency compared to the wild-type enzyme emphasizes the potential benefits of screening large libraries for novel catalytic activities. The catalytic activity of the isolated OmpT variants leading to the release of AMC from cleavage of substrate 2 is a fully consistent, albeit unexpected, solution to the selection criteria employed in the 96-well plate assay. This latter result indicates that alteration of certain protease substrate cleavage activities may not be limited to incremental changes, but may, in fact, be amenable to a more striking degree of modulation. Experiments are currently being planned to explore how far OmpT substrate specificity can be altered by directed evolution.

Although OmpT is a native surface enzyme, the strategy shown in Figure 1 can be employed with a wide variety of heterologous enzymes using well-established methods for protein surface display in *E. coli*, *Saccharomyces cerevisiae*, or *Streptococcus* spp.¹⁶ Current FACS technology allows the screening of 10^8 cells per hour, and the ability to screen quantitatively libraries of such size for catalytic activity may open new avenues to the directed evolution of enzyme substrate preferences, physical properties, and catalytic chemistry.

Experimental protocol

Plasmid construction. Unique site elimination was used to construct mutations in *ompT* (ref. 29) at codons 101 (pDS26) and 212 (pDS27), in each case changing from histidine to alanine. The *HindIII*-*MluI* site change primer 5'-CAGGCATCGACGGCTGGCGCTAATC-3' was used to construct all point mutations. Targeted mutations were made using primers 5'-GTG-TATCAGAGCTCTACTTTTATC-3' (H101A) and 5'-CCGGT-CATAAGCTTCATCGTTTATCAG-3' (H212A)²⁸. Changed bases are in bold-face type. All mutations were confirmed by dideoxynucleotide sequencing.

To transfer the native *ompT* and H212A *ompT* to a lower copy number background, the 2.0 kb *PstI*-*EcoRI* fragment from pML19 and pDS27,

Table 2. Mutational analysis of unique isolated clones selected for altered substrate specificity

	Position															
	33	87	111	131	137	141	149	152	186	200	240	276	278	284	286	288
WT	Glu	Met	Glu	Met	Ser	Phe	Ile	Ser	Ser	Tyr	Tyr	Ser	Asn	Asn	Asn	Ile
G11	Lys	Leu	Val	Lys	Asn	Ala	Val		Cys	Phe	Phe	Arg	Ile		Ser	Phe
C5																
B4																

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respectively, was cloned into pBR322. The resultant plasmids were called pDS318 (native ompT) and pDS327 (H212A ompT).

Substrate synthesis. FRET substrates 1 and 2 were synthesized by reacting the appropriate deprotected peptides (University of Texas Peptide Synthesis Facility) with the following reagents: (1) Tetramethylrhodamine iodoacetamide (Molecular Probes, Eugene, OR), dimethyl formamide (DMF); (2) BODIPY-FL-SE (Molecular Probes, Eugene, OR), DMF, according to the manufacturer's protocols. Substrates 1 and 2 were purified by medium-pressure preparative reverse-phase liquid chromatography (FPLC; Pharmacia, Piscataway, NJ), eluted with a 120 min linear gradient of acetonitrile (ACN) in H₂O with 0.7% trifluoroacetic acid (TFA). Compound 4 was purchased (Sigma Co., St. Louis, MO), and compound 5 was synthesized from valinyl-7-amido-4-methylcoumarin (9 mg, 32 μ mol) (Bachem, Bubendorf, Switzerland), Cbz-Arg-OH (10 mg, 32 μ mol) (Novabiochem, La Jolla, CA), PyBOP (33 mg, 64 μ mol) (Novabiochem, La Jolla, CA), N-methylmorpholine (14 μ l, 128 μ mol) (Aldrich, St. Louis, MO) in 1 ml anhydrous DMF, stirred at room temperature for 4 h. Purification was carried out using C18 Sep-Pak cartridges (Waters Corp., Milford, MA) eluted with ACN-H₂O mixtures. Yield was 14 mg (25 mmol, 78%) of compound 5 following lyophilization. Peptide 3 was purchased (Chiron, Clayton, Australia).

Flow cytometric analysis. UT5600 was transformed with either pDS26 or pML19 encoding the wild-type OmpT. Overnight cultures of UT5600, UT5600/pDS26, and UT5600/pML19 were washed with 1% sucrose, diluted to 0.01 OD₆₀₀, labeled for 10 min with 50 nM substrate 1 in 1% sucrose, diluted into 1 ml 1% sucrose, and analyzed using a Becton-Dickinson FACSsort.

Single-step enrichment of UT5600/pML319 from a 5,000 fold excess of cells expressing the OmpT H212A variant (UT5600/pDS327). Overnight cultures of UT5600/pML319 and UT5600/pDS327 were mixed at a ratio of 1:5,000 (UT5600/pDS327), labeled as above, and analyzed by FACS. A total of 159,715 cells of the labeled mixture were examined in 8.5 min. Thirty-two cells falling inside of RI were collected in 45 ml of sheath fluid, and were regrown overnight at 37°C in a total of 100 ml Luria-Bertani (LB) medium. Cells were harvested, washed with 1% sucrose, labeled with substrate 1 as above, and analyzed by FACS.

Library construction. A library of random mutants was constructed by error-prone PCR using 0.01, 0.15, 0.25, or 0.5 mM Mn²⁺ (ref. 31). The PCR product, pAMP1 vector DNA (Life Technologies, Rockville, MD), annealing buffer, and 2 units uracil DNA glycosylase in a total volume of 20 μ l were incubated for 40 min at 37°C, followed by 1 h at 4°C. Each reaction mixture was then electroporated into electrocompetent XL-1 Blue *Stratagene* cells, and serial dilutions were plated on selective plates to determine the number of independent transformants. The libraries were pooled, and the cells grown at 37°C overnight in LB medium with antibiotic selection. Plasmid DNA was isolated and subsequently transformed by electroporation into UT5600 for library screening.

Library screening. Transformants were grown at 37°C in LB medium, harvested at 16 h, washed with 1% sucrose, and reactions were prepared by incubating with 50 nM substrate 2 for 40 min. A 5 μ l aliquot of the reaction was then diluted into 1 ml 1% sucrose. FACS gates were set based upon FSC/SSC and FL1/FL2. A total of 1,929,783 cells were examined in 24 min, and 352 viable cells were collected. The collected solution was filtered, and the filters were placed on agar plates containing 100 μ g/ml carbenicillin. At 12 h, the 352 colonies were inoculated into minimal M63 medium containing amino acids and glycerol as the carbon source in quadruplicate 96-well plates. After 20 h of growth at 37°C, 20 μ l of substrate 4 or 5 and 5 U/ml aminopeptidase M (Sigma) were added to all wells, and plates were incubated at 37°C for 16 h. Fluorescence intensities were measured on a Bio-Tek FL600 (Bio-Tek Instrument, Winooski, VT) fluorescence plate reader, with excitation at 360/40 and emission at 460/40. Optical densities were also measured at 600 nm to correct for cell growth effects. All liquid handling steps were performed with a Beckman Biomek 2000 robotic station (Beckman Instruments, Fullerton, CA).

Enzyme purification and kinetic analysis. OmpT was isolated as previously described³⁴, and 1.5 μ g of purified enzyme (80% by SDS-PAGE) was incubated with 20 μ M substrate 4 or 5, and the products were analyzed by high-performance liquid chromatography (HPLC) on a Ydaci C₁₈ column using 5% ACN/95% H₂O for 1 min, gradually increasing to 55% ACN/95% H₂O by 31 min, 95% ACN/5% H₂O for an additional 5 min, returning to 5% ACN/95% H₂O over 5 min, followed by 5 min at 55% ACN/95% H₂O. Cleavage kinetics for compound 3 with wild-type OmpT and the variants were performed using the same HPLC conditions as above, and substrate concentrations varied from 20 μ M to 300 μ M. Apparent cleavage rates were determined by monitoring the amount of cumulative products produced as a function of substrate peptide concentration, using a hyperbolic regression analysis to determine apparent K_m and K_d values. Cleavage products were determined by

liquid chromatography-mass spectrometry (LC-MS) (Echelleite Spectrophotograph and Images; ESI) as well as matrix-assisted laser desorption/ionization mass spectral (MALDI-MS) analysis. The product ratios were determined by HPLC, using the integrated peak areas monitored at 280 nm.

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